

Optimized peripheral nerve decellularization protocol with enhanced extracellular matrix integrity, mechanical strength, and biocompatibility vs Hudson's standard method

Hussein Abboud Shanan Al-Shafeay¹, Saber Zahri^{1*}, Asadollah Asadi¹, Arash Abdolmaleki²

¹ Department of Biology, Faculty of Science, University of Mohaghegh Ardabili, Ardabil, Iran

² Department of Biophysics, Faculty of Advanced Technologies, University of Mohaghegh Ardabili, Namin, Iran

ARTICLE INFO

Article type:

Original

Article history:

Received: May 2, 2026

Accepted: Sep 22, 2026

Keywords:

Biocompatibility

Caspase-3

Decellularization

Extracellular matrix

Hudson's method

Optimized protocol

Sciatic nerve

ABSTRACT

Objective(s): Decellularized nerve scaffolds are promising biomaterials for peripheral nerve regeneration. This study compared an optimized decellularization protocol with Hudson's standard method to assess structural, mechanical, biochemical, and biological performance.

Materials and Methods: Rat nerve tissues underwent native and optimized decellularization protocols. Morphological evaluation was performed using H&E and DAPI staining, while biomechanical properties and degradation kinetics were assessed over 28 days. DNA content, S100B, NEFL, collagen, laminin, and glycosaminoglycans (GAGs) were quantified to evaluate extracellular matrix (ECM) preservation and cellular removal. Neurotrophic factors, including NGF, BDNF, and GDNF, were measured to assess regenerative potential. Biocompatibility was evaluated after transplantation of rat bone marrow-derived mesenchymal stem cells (BM-MSCs) using MTT and Caspase-3 assays. All experiments used n=5 specimens per group, with triplicate measurements.

Results: The optimized protocol achieved near-complete decellularization while preserving ECM architecture and significantly outperformed Hudson's method ($P<0.001$). Optimized scaffolds exhibited intermediate stiffness (13.76 MPa), higher ultimate strength (2.3 MPa vs 1.62 MPa; $P<0.01$), and slower degradation (48.3% reduction; $P<0.001$). DNA content decreased by 96.9% in the optimized group compared with 90.5% in Hudson's group. Neurotrophic factors were better preserved, with NGF reaching 56.2 pg/mg ($P<0.001$), and ECM components such as collagen and laminin improved significantly ($P<0.05$). Optimized scaffolds enhanced cell viability (OD 0.58 vs 0.43; $P<0.001$) and reduced Caspase-3 activity ($P<0.01$).

Conclusion: The optimized decellularization protocol surpasses Hudson's method by achieving effective decellularization while preserving ECM structure, mechanical integrity, and BM-MSC viability, thereby supporting its potential application in peripheral nerve tissue engineering.

► Please cite this article as:

Al-Shafeay HASH, Zahri S, Abdolmaleki A, Asadi A. Optimized peripheral nerve decellularization protocol with enhanced extracellular matrix integrity, mechanical strength, and biocompatibility vs Hudson's standard method. Iran J Basic Med Sci 2026; 29: 1902-1911. doi: <https://dx.doi.org/10.22038/ijbms.2026.95387.20565>

Introduction

Traumatic peripheral nerve injuries (PNIs) are a significant subset of nerve trauma, mainly affecting young adult males, with an incidence of about 1.3% among trauma patients. These injuries typically result from sharp trauma or blunt force, primarily impacting the upper extremities and leading to sensory loss, motor impairment, and chronic pain (1-3). The complexity of nerve anatomy and injury mechanisms complicates clinical management and often requires timely surgical intervention for better outcomes (4, 5). Despite these advances, many patients experience incomplete recovery and long-term disability, underscoring the need for improved therapeutic strategies, such as optimized decellularized nerve scaffolds to enhance nerve regeneration (6, 7).

Peripheral nerves possess an intrinsic ability to regenerate after injury (8). However, this capability is insufficient in severe traumatic injuries that result in lost

nerve tissue. In such cases, spontaneous regeneration does not occur, so nerve gap reconstruction is needed to restore function. The current gold standard for such reconstruction is autologous nerve grafting, which involves transplanting the patient's own nerve tissue (9, 10). Despite its efficacy, autografts have significant limitations, including donor site morbidity, limited availability of suitable donor nerves, and often incomplete functional recovery. These challenges underscore the urgent need for alternative strategies that overcome autograft limitations (1, 11). Tissue engineering has introduced decellularized nerve scaffolds that provide a native extracellular matrix (ECM) as a biological framework for nerve regeneration, without the risk of immune reactions (12, 13). These scaffolds promise improved repair outcomes and expanded treatment options for severe peripheral nerve injuries, addressing the critical need for effective nerve repair solutions (14, 15).

Decellularized nerve scaffolds offer significant advantages

*Corresponding author: Saber Zahri. Department of Biology, Faculty of Science, University of Mohaghegh Ardabili, Ardabil, Iran. Email: Zahri@uma.ac.ir



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over synthetic alternatives, as they preserve the native ECM architecture and key bioactive cues for nerve regeneration (16). Various decellularization methods, including chemical, enzymatic, and physical techniques, aim to remove cellular components while maintaining ECM integrity (17-19). Common approaches include detergents such as Triton X-100, enzyme treatments such as trypsin, and freeze-thaw cycles, each of which affects tissue preservation differently (20, 21). One significant benefit of these scaffolds is their biocompatibility, which reduces the risk of immune rejection and supports cell attachment, migration, and axonal growth. Additionally, native tissue better mimics the physiological microenvironment than synthetic constructs (16, 22). However, challenges persist, including incomplete cellular removal and potential damage to ECM components, which can impede regeneration. Ongoing research is essential to optimize decellularization protocols, enhance ECM preservation, and improve the functional properties of these scaffolds for nerve repair.

Building on the limitations of current decellularization protocols, including the well-known Hudson's method, this study aims to develop and comprehensively characterize an optimized decellularization protocol for peripheral nerve scaffolds that achieves complete cellular removal while preserving ECM integrity and bioactivity. Our objective was to integrate mechanical, chemical, and enzymatic approaches within a multi-stage process to minimize ECM disruption, retain neurotrophic factors, and maintain the scaffold's mechanical resilience. The optimized protocol was systematically compared to Hudson's method in terms of structural preservation, biochemical composition, mechanical performance, degradation kinetics, and cellular biocompatibility, using both histological and molecular assays. Ultimately, this study seeks to establish a next-generation decellularization approach that provides superior ECM preservation, enhanced biomechanical functionality, and improved biocompatibility, thereby offering an advanced platform for peripheral nerve tissue engineering and regeneration.

Materials and Methods

Ethics statement

All experimental procedures involving rat and human peripheral nerve tissues were conducted in accordance with the EU Council Directive of 24 November 1986 and approved by the Ethics Committee of Mohaghegh Ardabili University. All protocols adhered to institutional, national, and international guidelines for the ethical use of biological tissues.

Chemicals

Critical reagents were procured from established manufacturers: sulfobetaine-10 (SB-10) from Alfa Aesar (USA); sulfobetaine-16 (SB-16) from TCI Chemicals (Japan); Triton X-100, papain, chondroitin sulfate, Harris hematoxylin, MTT, Tris-HCl, EDTA, RIPA buffer, and peracetic acid from Sigma-Aldrich (USA); sodium acetate, ethanol, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and eosin from Merck (Germany); DNase I from Roche (Switzerland); RNase A and DAPI from Thermo Fisher Scientific (USA); proteinase K from Qiagen (Germany); Quant-iT™ PicoGreen® dsDNA Kit from Invitrogen (USA); DMEM from Gibco (USA); ELISA kits for growth factors from R&D Systems (USA). Zwittergent 10 was sourced from MilliporeSigma (USA) to

ensure reagent consistency across protocols.

Acellular nerve preparation

Rat sciatic nerves (15 mm length) were harvested from male Wistar rats and rinsed in sterile phosphate-buffered saline (PBS, pH 7.4) to remove blood residues. Samples were mechanically disrupted by sonication in 1 M NaCl at 4 °C for 4 min. Two cycles of osmotic shock were performed by alternating immersion in 1 M NaCl and distilled water at 4 °C. Enzymatic digestion was performed in PBS at 37 °C for 18 min using a solution containing papain (2 mg/ml), cysteine (5 mM), EDTA (0.5 mM), albumin (1%), and CaCl₂ (1 mM), followed by inhibition with 10% fetal bovine serum. Chemical decellularization involved sequential incubation in CHAPS (0.5%) and Zwittergent 10 (0.1%) detergents at 4 °C for 5 hr each, with PBS washes between steps. Nucleic acid removal was achieved by treating with DNase I (50 U/ml) and RNase A (1 mg/ml) in Tris-HCl buffer (pH 7.5) containing Mg²⁺ and Ca²⁺ ions at 37 °C for 5 hr, with the solution refreshed after 3 hr. Final washes were performed in PBS with EDTA (0.1%) at room temperature. Samples were sterilized by UV-C irradiation (254 nm) for 30 min per side at a distance of 10 cm with continuous rotation. Decellularized scaffolds were stored at 4 °C in PBS supplemented with penicillin (100 U/ml) and gentamicin (50 µg/ml). For control experiments, decellularized nerve scaffolds were generated using the detergent-based procedure described by Hudson et al. (2004) (15). Briefly, harvested nerves were immersed in distilled deionized water for 7 hr with continuous agitation on a vertical rotator at 25 °C. The tissue was then transferred to a 125 mM sulfobetaine-10 (SB-10) buffer containing phosphate and sodium salts for 15 hr. After a brief rinse with a phosphate-sodium washing solution, the nerves were exposed to 0.14% Triton X-200 and 0.6 mM SB-16 for 24 hr. This was followed by another wash, a second 7-hr SB-10 treatment, and a 15-hr cycle in the SB-16/Triton X-200 solution. Finally, the decellularized constructs were thoroughly rinsed with buffer and stored in the same solution at 4 °C until use.

Morphological assessment

Histological staining

Histological assessments were performed using three complementary staining approaches. For general morphological analysis, paraffin-embedded tissue sections were deparaffinized in xylene (2×5 min), rehydrated through a graded ethanol series (100% to 70%), and stained with Harris hematoxylin (5 min) followed by differentiation in acid alcohol (1%) and bluing in alkaline water. Counterstaining with 1% eosin (1–3 min) was followed by dehydration in ethanol and xylene clearing, before coverslipping. For nuclear visualization, paraformaldehyde-fixed samples were permeabilized with 0.1% Triton X-100 (10 min), incubated with 300 nM DAPI (5 min, dark), and imaged using fluorescence microscopy with a DAPI-specific filter set after anti-fade mounting. All procedures adhered to standardized protocols for histological and cytological characterization (23).

Biomechanical characterization

Decellularized sciatic nerve segments (n=5) were precision-sectioned to 10 mm gauge length using micro-dissection tools. Following 60-minute PBS equilibration at 22±1 °C, cross-sectional area was calculated by measuring

the major and minor diameters at three points along each specimen using digital caliper microscopy (resolution: 1 μ m). An elliptical geometry was assumed ($\text{Area} = \pi \times a \times b$, where a and b are the semi-major and semi-minor axes), which approximates the native elliptical shape of the rat sciatic nerve and is standard in peripheral nerve biomechanical studies. Uniaxial testing was conducted on a SANTAM-STM₂₀ system (10 N load cell, ISO 37-compliant grips) at a displacement rate of 1 mm/min. Nerve segments were aligned along the longitudinal axis of the nerve fibers during mounting and testing to ensure physiologically relevant tensile measurements. Specimens underwent three preconditioning cycles (0-0.1 N) before final ramp-to-failure testing. Continuous data acquisition at 100 Hz captured load-displacement curves to determine Young's modulus (MPa), ultimate stress (MPa), ultimate strain (%), and peak extension (mm). To prevent dehydration-induced stiffening, specimens were kept continuously hydrated during the entire testing procedure by regular PBS spraying (24).

Degradation testing

PBS-based degradation was used to provide a standardized, reproducible condition for comparing relative hydrolytic stability between protocols, following established methods for acellular scaffold characterization. Decellularized rat sciatic nerve segments (10 mm, $n=5$) were incubated in PBS (pH 7.4) at 37 °C under static conditions to simulate physiological hydrolysis for 28 days. Initial dry weights (W_0) were recorded using an analytical balance (± 0.01 mg precision). At weekly intervals (days 7, 14, 21, 28), specimens were removed from PBS, oven-dried at 55 °C until completely dry, and re-weighed (W_t) to calculate mass loss using the formula: $(W_0 - W_t) / W_0 \times 100$. All measurements were performed in triplicate per sample. At each time point, specimens were removed from PBS, rinsed three times with distilled water to remove residual salts, and oven-dried at 55 °C until constant weight was achieved. Additionally, the PBS was replaced weekly (days 7, 14, and 21) to prevent degradation by-product buildup and maintain consistent experimental conditions during the 28-day incubation period.

Elimination of cellular proteins and DNA

S100B is a well-established Schwann cell marker, while NEFL (neurofilament light chain) marks neuronal axons. Quantifying these markers in decellularized tissue provides an indirect measure of residual cellular and axonal components after treatment. Fresh native and decellularized rat sciatic nerves were homogenized at 100 mg/ml in RIPA buffer with protease inhibitors for S100B analysis. S100B and NEFL were measured in separate supernatant preparations to avoid cross-interference. For NEFL quantification, 0.5 M urea was added only for this analysis. S100B was measured in supernatants prepared without urea, ensuring that urea did not affect S100B stability or quantification. The samples were incubated on ice for 1 hr with agitation and then subjected to three freeze-thaw cycles (-20 °C). After centrifugation ($12,000 \times g$, 15 min, 4 °C), the supernatants were analyzed using ELISA kits for S100B (ab234573, Abcam) and NEFL (ab288182, Abcam) according to the manufacturer's protocols. Absorbance was measured at 450 nm, with blank subtraction and standard curve normalization. Native nerve samples served as baseline references to quantify S100B and NEFL clearance

after decellularization, with results expressed as percentage reduction relative to native tissue levels.

DNA quantification

Lyophilized sciatic nerve specimens (20 mg dry weight, $n=5$ /group) underwent enzymatic digestion in a lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 0.5% SDS, 10 mM EDTA) supplemented with 1.5 mg/ml Proteinase K at 55 °C for 18 hr. After digestion, samples were vortex-mixed and centrifuged at $12,000 \times g$ for 20 min at 4 °C. Supernatants were diluted to 0.3 volumes with 3 M sodium acetate (pH 5.2) and chilled at -20 °C before a repeat centrifugation to remove residual proteins and lipids that precipitate during cold incubation. Nucleic acids were precipitated using 2.2 volumes of chilled 95% ethanol and incubated at -20 °C. The resulting DNA pellet was washed twice with 70% ethanol, air-dried for 10 min in a laminar flow hood with continuous air circulation at room temperature, and rehydrated in 50 μ l molecular-grade water at 65 °C. Quantification was performed using a NanoDrop OneC spectrophotometer for A260/A280 ratios. DNA content was normalized to the dry weight of samples and reported as ng DNA per mg dry tissue (25).

ECM quantification

Collagen content was quantified using a hydroxyproline assay kit; tissue samples were acid-hydrolyzed (6 M HCl at 110 °C for 24 hr) and neutralized with 10 M NaOH (approximately 0.6 ml per 1 ml of hydrolysate). Hydroxyproline oxidation with chloramine-T and reaction with *p*-dimethylaminobenzaldehyde (DMAB) produced a chromophore measured spectrophotometrically at 557 nm, calibrated against standards (0.1–10 μ g/ml). Non-collagenous hydroxyproline interference was controlled with a tissue-free hydrolysis blank containing all hydrolysis reagents (6 M HCl) but no tissue. The blank was processed identically through hydrolysis, neutralization, and the colorimetric assay, allowing subtraction of background absorbance from reagents and non-specific color development. For glycosaminoglycan (GAG) analysis, sciatic nerve specimens (16 mg; $n=5$) were enzymatically digested in 400 μ l lysis buffer and incubated at 65 °C for 16 hr. Following centrifugation at $6,000 \times g$ for 15 min at 4 °C, supernatants were collected, mixed with a chromogenic GAG reagent, and absorbance was measured at 525 nm. Calibration curves were used to quantify GAG in μ g/mg dry tissue mass. Laminin detection via Laminin ELISA Kit (ab213774) involved normalizing tissue lysates to 1 mg/ml total protein, probing with antibodies, and quantifying absorbance at 450 nm against recombinant laminin standards. All assays included triplicate replicates, normalized to dry tissue mass (mg).

Neurotrophic factor assessment

All measurements were performed using commercial ELISA kits from Abcam, including nerve growth factor (NGF) Kit (ab193736), brain-derived neurotrophic factor (BDNF) Kit (ab213899), and glial cell line-derived neurotrophic factor (GDNF) Kit (ab213901), following the manufacturer's instructions. All kits utilize a sandwich ELISA format with pre-coated capture antibodies and biotinylated detection antibodies, followed by streptavidin-HRP conjugate and TMB substrate. Decellularized nerves were lyophilized, homogenized in ice-cold RIPA buffer with protease inhibitors, and centrifuged at $12,000 \times g$ for 15 min

at 4 °C. The supernatants were used to measure concentrations of Neurotrophic Factor. Samples and standards were added in duplicate to precoated 96-well plates and incubated. After washing, detection antibodies and enzyme conjugates were added, followed by the substrate solution. Absorbance was read at 450 nm, and growth factor concentrations were calculated from standard curves and normalized to dry tissue weight (pg/mg). All assays were performed in triplicate.

Biocompatibility

Recellularization and MTT assay

Rat bone marrow-derived mesenchymal stem cells (BM-MSCs) at passage 4 were expanded in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin under standard culture conditions (37 °C, 5% CO₂). For scaffold seeding, BM-MSCs were trypsinized and resuspended at 1×10^6 cells/ml. Aliquots of 20 μ l (2×10^4 cells) were pipetted onto each scaffold as the initial seeding density, with 2 hr for attachment before adding 200 μ l of complete medium. Cell viability was quantified via MTT assay at 24, 48, and 72 hr by transferring scaffolds to new plates, incubating with 20 μ l of MTT solution at a concentration of 0.5 mg/ml (4 hr, 37 °C), and dissolving formazan crystals in 200 μ l DMSO. Absorbance was measured at 570 nm, with viability expressed as a percentage of control values. The test used three biological samples, each with three replicates (26).

Caspase-3 activity assay

Caspase-3 activity was measured in BM-MSCs using the Caspase-3 Assay Kit (Colorimetric, Abcam, ab39401) according to the manufacturer's instructions. Scaffolds were placed in 96-well flat-bottom plates, and cells were seeded directly onto the scaffold surface. After 2 hr of initial attachment, 200 μ l of complete medium was added to each well. Protein concentration was measured using the BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) according to the manufacturer's instructions. Samples containing equal amounts of protein (50 μ g) were incubated with the caspase-3 substrate Ac-DEVD-pNA at 37 °C for

2 hr. Cleavage of the substrate by active caspase-3 released the chromophore p-nitroaniline (pNA), and absorbance was measured at 405 nm using a microplate reader. All experiments were performed in triplicate.

Statistical analysis

Quantitative data are presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using SPSS 27, and normality was assessed using the Shapiro-Wilk test. Group differences were analyzed using one-way ANOVA with Tukey's *post hoc* test; $P < 0.05$ was considered significant.

Results

Morphology

The morphological evaluation revealed distinct differences in cellular clearance and ECM preservation across decellularization methods. H&E staining demonstrated intact cellular architecture in native nerves (Figure 1A), characterized by visible nuclei and cytoplasmic eosinophilic staining. In contrast, Hudson's method showed residual nuclear debris and disrupted endoneurial structures (Figure 1B), whereas the optimized protocol achieved complete decellularization with preserved ECM organization (Figure 1C). The optimized approach maintained basal lamina integrity and collagen alignment, outperforming Hudson's method in structural continuity.

DAPI fluorescence analysis showed intense, uniformly distributed nuclear signals throughout native nerves (Figure 2A), consistent with dense cellularity. Scaffolds processed via Hudson's method retained fragmented DNA remnants with weak, scattered, punctate fluorescence (Figure 2B), indicating incomplete removal of nuclear material. In contrast, the optimized protocol showed no detectable DAPI fluorescence (Figure 2C), confirming near-complete elimination of all nuclear material.

Biomechanical characteristics

Biomechanical characterization revealed significant intergroup variation ($P < 0.001$). As shown in Figure 3A, the optimized decellularization protocol yielded an intermediate Young's modulus (13.76 MPa) between native nerve tissue (17.1 MPa) and Hudson's method (9.44 MPa), indicating superior tensile strength compared to Hudson's method ($P < 0.001$). Ultimate stress analysis showed that the

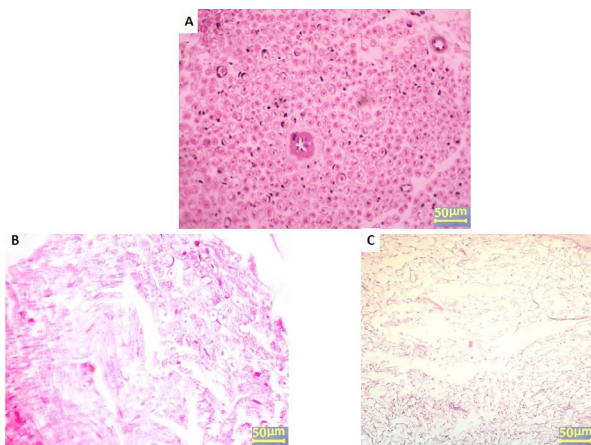


Figure 1. Hematoxylin and eosin (H&E) staining of rat nerve tissues (scale bars: 100 μ m)

(A) Native nerve showed intact cellular architecture, with visible Schwann cell nuclei (dark blue/purple, elongated along the longitudinal axis), axonal structures surrounded by eosinophilic cytoplasm, and organized endoneurial collagen fibers oriented longitudinally along the nerve axis. (B) Hudson's method shows residual nuclear debris (dark, fragmented punctate staining) and disrupted endoneurial structures, with loss of organized collagen alignment and compromised structural continuity. (C) The optimized decellularization protocol achieves complete decellularization while preserving ECM organization, including intact basal lamina and aligned collagen fibrils (pink/red eosinophilic staining), demonstrating better structural continuity than Hudson's method.

ECM: Extracellular matrix

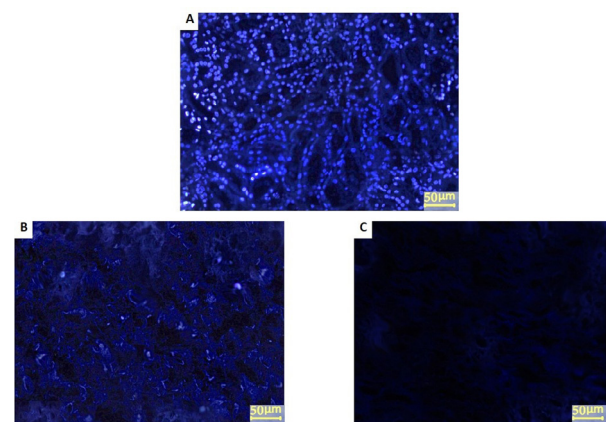


Figure 2. 4, 6-diamidino-2-phenylindole (DAPI) fluorescence analysis of nucleic acid removal (scale bars: 100 μ m)

(A) Native nerve shows intense, uniform nuclear signals throughout the tissue section. (B) Hudson's method shows residual punctate fluorescence, indicating fragmented DNA remnants scattered within the tissue. (C) The optimized decellularization protocol achieves near-complete removal of nuclear material with no detectable DAPI fluorescence, confirming thorough nucleic acid elimination.

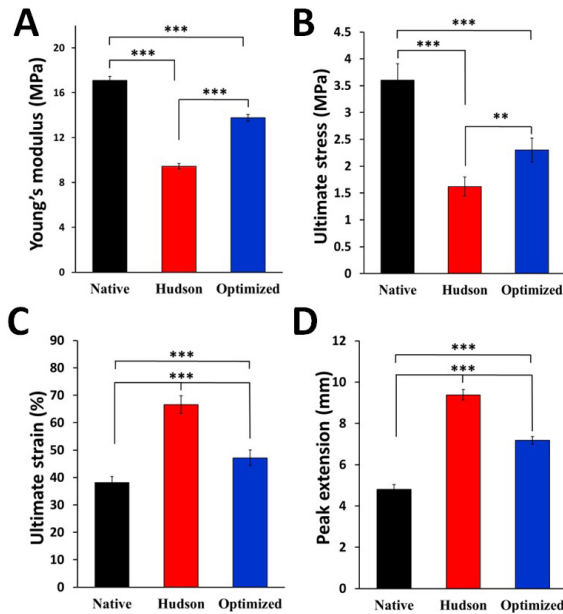


Figure 3. Biomechanical characterization of decellularization methods (A) Young's modulus: native nerve 17.1 MPa, Hudson's method 9.44 MPa, optimized protocol 13.76 MPa (significantly higher than Hudson's). (B) Ultimate stress: native tissue 3.6 MPa, optimized 2.3 MPa, Hudson's 1.62 MPa (optimized reaches 63.9% of native strength). (C) Ultimate strain: Hudson's method 66.6% (74.3% more than native 38.2%), optimized 47.2% (closer to native). (D) Peak extension: optimized 7.18 mm, Hudson's 9.38 mm, native 4.8 mm. Data were analyzed using One-Way ANOVA and Tukey *post hoc* tests. Results are presented as mean±SD (n=5), with statistical significance indicated by ***P*<0.01 and ****P*<0.001.

optimized protocol (2.3 MPa) achieved 63.9% of the native tissue strength (3.6 MPa) and significantly outperformed Hudson's method (1.62 MPa, *P*<0.01), suggesting enhanced structural integrity (Figure 3B). Paradoxically, Hudson's method exhibited hyperelastic behavior, with 74.3% greater ultimate strain than native controls (66.6% vs 38.2%, *P*<0.001). In contrast, the optimized protocol maintained deformation capacity (47.2% strain) closer to native characteristics (Figure 3C). Peak extension measurements further confirmed these trends, with the optimized group (7.18 mm) showing a 49.6% reduction in excessive extensibility compared to Hudson's method (9.38 mm) while remaining more compliant than native tissue (4.8 mm, *P*<0.001) (Figure 3D). Overall, the optimized scaffold's stress-strain behavior was intermediate between native and Hudson-treated nerves, with a more physiological elastic region than the hyperelastic behavior observed in Hudson's method.

Degradation profile

The degradation kinetics differed significantly among

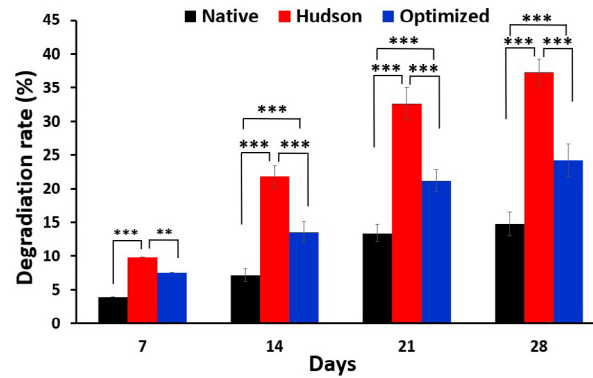


Figure 4. Degradation profile of decellularized nerve scaffolds over 28 days showed that native tissue degraded from 4.0% on Day 7 to 14.8% on Day 28. Hudson's method had greater mass loss, from 9.8% to 37.2%. The optimized protocol displayed more controlled degradation: 7.6% on Day 7, 13.6% on Day 14, 21.2% on Day 21, and 24.2% on Day 28. It degraded 48.3% slower than Hudson's method and 63.2% faster than native tissue. Data were analyzed using One-Way ANOVA and Tukey *post hoc* tests. Results are presented as mean±SD (n=5), with statistical significance indicated by ***P*<0.01 and ****P*<0.001.

groups over the 28-day incubation period (Figure 4). Native tissue degraded gradually from 4.0±0.3% on Day 7 to 14.8±1.1% on Day 28. Hudson's method showed faster mass loss, increasing from 9.8±0.7% on Day 7 to 37.2±2.4% on Day 28. The optimized protocol showed controlled degradation, with intermediate values at all time points: 7.6±0.5% (Day 7), 13.6±0.9% (Day 14), 21.2±1.3% (Day 21), and 24.2±1.6% (Day 28). It degraded significantly more slowly than Hudson's method at all time points (*P*<0.01 for Day 7; *P*<0.001 for Days 14, 21, and 28), while degrading significantly faster than native tissue (*P*<0.01 at Day 7; *P*<0.001 at Days 14, 21, and 28). At Day 28, cumulative mass loss in the optimized group (24.2±1.6%) was 34.9% lower than Hudson's method and 63.5% higher than native tissue, indicating a favorable profile for tissue remodeling applications.

Elimination of cellular proteins and DNA

DNA content analysis showed a progressive reduction across experimental groups, with native tissue containing 596.2 ng/mg total DNA. Hudson's decellularization method achieved a 90.5% reduction (56.8 ng/mg), while the optimized protocol showed superior efficiency with a 96.9% reduction (18.4 ng/mg) (*P*<0.001). This represents an additional 67.6% reduction compared to Hudson's method, indicating improved elimination of nuclear material and reduced immunogenicity (Figure 5A).

Native tissue showed S100B levels at 18.8 ng/mg. Hudson's decellularization method reduced this by 91.1% to

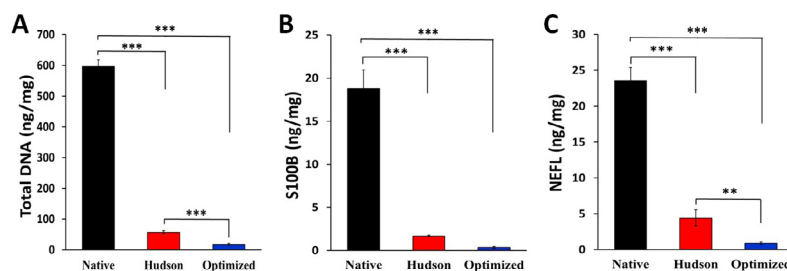


Figure 5. Cell protein and DNA elimination across decellularization methods (A) DNA content: native tissue 596.2 ng/mg; Hudson's method reduces to 56.8 ng/mg (90.5% reduction); optimized protocol reduces to 18.4 ng/mg (96.9% reduction). (B) S100B levels: native tissue 18.8 ng/mg; Hudson's method 1.68 ng/mg; optimized protocol 0.36 ng/mg. (C) NEFL content: native tissue 23.6 ng/mg; Hudson's method 4.4 ng/mg; optimized protocol 0.9 ng/mg. The optimized protocol achieves greater reductions in immunogenic components than Hudson's method, enhancing biocompatibility. Data were analyzed using One-Way ANOVA and Tukey *post hoc* tests. Results are presented as mean±SD (n=5), with statistical significance indicated by ***P*<0.01 and ****P*<0.001.

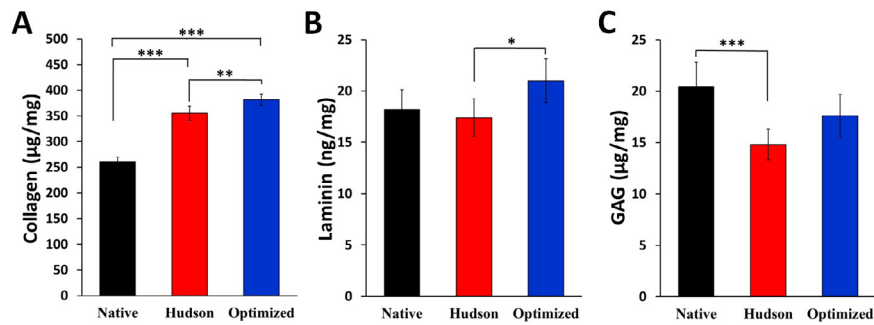


Figure 6. Evaluation of ECM preservation across decellularization protocols

(A) Collagen content increases from Hudson's method (355 µg/mg) to the optimized protocol (381.6 µg/mg). (B) Laminin is better preserved in the optimized protocol (21 ng/mg) than in Hudson-treated samples (17.4 ng/mg). (C) GAG content shows partial recovery with the optimized protocol (17.6 µg/mg) compared with Hudson's treatment (14.8 µg/mg), but the difference was not statistically significant. The higher collagen and laminin concentrations in decellularized tissue vs native nerve reflect removal of cellular and lipid material (which reduces tissue mass) and consequent concentration effects. Data were analyzed using One-Way ANOVA and Tukey *post hoc* tests. Results are presented as mean±SD (n=5), with statistical significance indicated by * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

ECM: Extracellular matrix; GAG: Glycosaminoglycan

1.68 ng/mg, while the optimized protocol achieved a 98.1% decrease to 0.36 ng/mg, both surpassing the 90% reduction threshold. The optimized method achieved a further 78.6% reduction compared with Hudson's method. However, this difference was not statistically significant (Figure 5B). NEFL content decreased significantly from 23.6 ng/mg in native tissue to 4.4 ng/mg (81.4% reduction) with Hudson's method ($P<0.001$) and to 0.9 ng/mg (96.2% reduction) with the optimized protocol (Figure 5C; $P<0.001$).

ECM preservation

Quantitative evaluation revealed distinct preservation patterns across decellularization protocols. Collagen content increased progressively from Hudson's method (355 µg/mg/dry tissue) to the optimized protocol (381.6 µg/mg/dry tissue) (Figure 6A; $P<0.01$). Laminin levels were better preserved in the optimized group (21 ng/mg/dry tissue) than in Hudson-treated samples (17.4 ng/mg/dry tissue) (Figure 6B; $P<0.05$). GAG analysis showed partial recovery with the optimized protocol (17.6 µg/mg/dry tissue) compared with Hudson's reduction (14.8 µg/mg/dry tissue). However, the difference was not statistically significant (Figure 6C). The higher collagen and laminin concentrations in decellularized tissue than in native nerve result from removing cellular and lipid material, which reduces tissue mass and increases the concentration of extracellular matrix components, not from new collagen synthesis.

Neurotrophic factor preservation

The optimized decellularization protocol demonstrated significant improvements in neurotrophic factor retention compared with Hudson's method. NGF concentrations increased 2.5-fold from 22.4 pg/mg in Hudson-processed samples to 56.2 pg/mg in the optimized protocol, representing 21.7% of native tissue levels (259.4 pg/mg) (Figure 7A; $P<0.001$). BDNF preservation doubled from 38.4 pg/mg to 77 pg/mg, recovering 20.8% of baseline concentrations observed in native tissue (370.8 pg/mg) (Figure 7B; $P<0.001$). GDNF retention improved 2.2-fold from 22.6 pg/mg to 49.4 pg/mg, equivalent to 17% of native tissue values (290 pg/mg) (Figure 7C; $P<0.001$). While the optimized protocol achieved substantial percentage improvements over Hudson's method, all measured neurotrophic factors remained markedly below native tissue benchmarks.

Cellular viability analysis

The results from BM-MSC transplantation onto scaffolds, with a control group on tissue culture polystyrene, were evaluated at 24, 48, and 72 hr using the MTT assay. Results revealed distinct temporal patterns in metabolic activity among experimental groups. Controls exhibited progressive increases in OD from 0.309 (24 hr) to 0.718 (72 hr), establishing baseline proliferation kinetics. Scaffold groups showed protocol-dependent responses, with the optimized

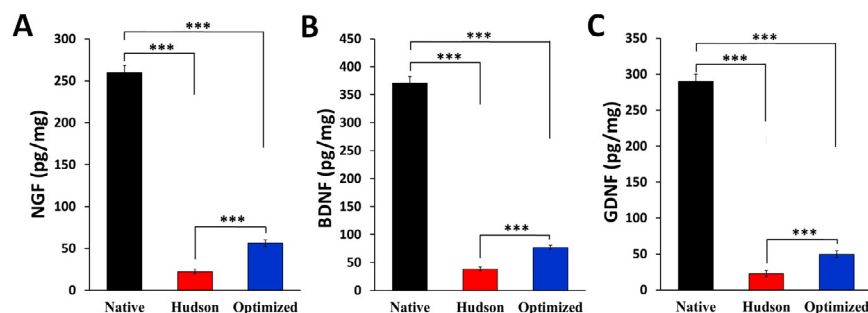


Figure 7. Neurotrophic factor retention across decellularization methods

(A) NGF: Hudson 22.4 pg/mg, while the optimized protocol achieved 56.2 pg/mg, representing 21.7% of the native tissue level (259.4 pg/mg). (B) BDNF: The optimized protocol increased this to 77 pg/mg, recovering 20.8% of the native baseline (370.8 pg/mg). (C) GDNF: Hudson 22.6 pg/mg, while the optimized protocol raised this to 49.4 pg/mg, equivalent to 17% of the native tissue level (290 pg/mg). Data were analyzed using One-Way ANOVA and Tukey *post hoc* tests. Results are presented as mean±SD (n=5), with statistical significance indicated by *** $P<0.001$.

NGF: nerve growth factor; BDNF: Brain-derived neurotrophic factor; GDNF: Glial cell line-derived neurotrophic factor

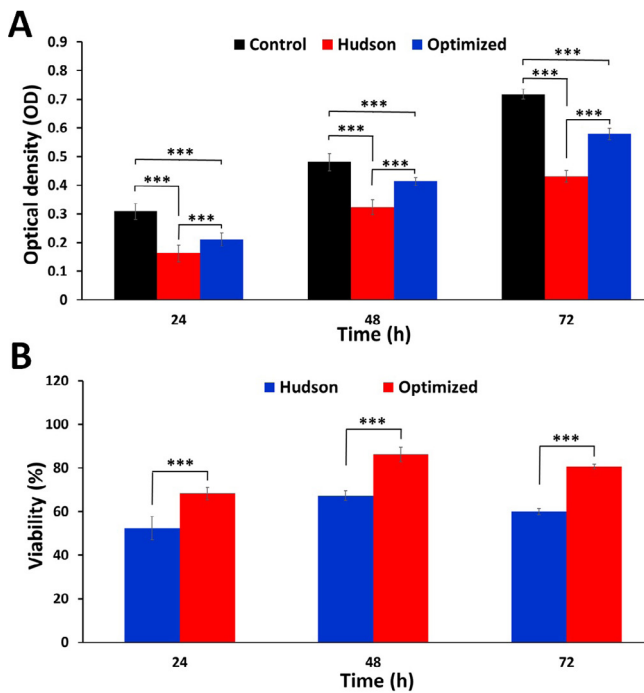


Figure 8. Metabolic activity and viability of BM-MSCs on scaffolds over 72 hr (A) MTT assay OD values: control (tissue culture polystyrene) increased from 0.309 at 24 hr to 0.718 at 72 hr. Scaffold groups showed protocol-dependent responses, with the optimized decellularization method yielding a higher OD at 72 hr (0.58) than controls (0.43). (B) Viability over time: the optimized protocol showed greater viability than Hudson over 24, 48, and 72 h. At 24 hr, viability was 68.24% (Optimized) vs 52.40% (Hudson); at 48 hr, 86.24% vs 67.32%; at 72 hr, 80.63% vs 60.00%. Data were analyzed using One-Way ANOVA and Tukey *post hoc* tests. Results are presented as mean±SD (n=10), with statistical significance indicated by *** $P<0.001$. BM-MSCs: Bone marrow-derived mesenchymal stem cells

decellularization method performing best. At 72 hr, optimized scaffolds reached 0.58 OD, compared with 0.43 OD in controls (Figure 8A; $P<0.001$). Viability analysis also showed significant differences between decellularization protocols over 72 hr. At 24 hr, the viability was 68.24% for the Optimized protocol compared to 52.40% for the Hudson protocol; at 48 hr, 86.24% (Optimized) versus 67.32% (Hudson); and by 72 hr, 80.63% (Optimized) versus 60.00% (Hudson) (Figure 8B; $P<0.001$).

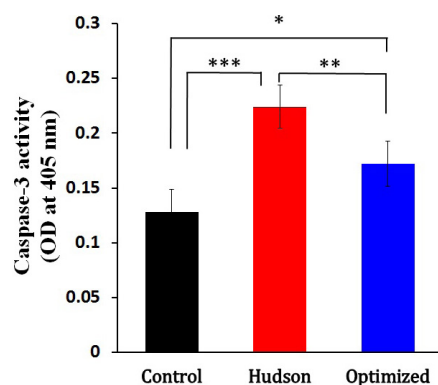


Figure 9. Caspase-3 activity. OD was measured at 405 nm for caspase-3 activity in cells exposed to scaffolds. At the time of measurement, Caspase-3 values were 0.128 (Control, tissue culture polystyrene), 0.224 (Hudson), and 0.172 (Optimized). The Hudson scaffold showed greater apoptotic-like activity than both Control and Optimized, with Optimized showing intermediate levels closer to those of Control. Data were analyzed using One-Way ANOVA and Tukey *post hoc* tests. Results are presented as mean±SD (n=5), with statistical significance indicated by * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.

Caspase-3 activity

Caspase-3 activity was assessed by measuring OD at 405 nm in cells exposed to scaffolds, with the control group cultured on tissue culture polystyrene (Figure 9). At the measurement point, Caspase-3 activity values were 0.128 for control, 0.224 for Hudson, and 0.172 for Optimized, indicating higher apoptotic-like activity in the Hudson scaffold relative to control ($P<0.001$) and Optimized ($P<0.01$). In contrast, the optimized scaffold showed intermediate activity closer to the control ($P<0.05$).

Discussion

In this study, we systematically compared an optimized decellularization protocol against the established Hudson's method and native nerve tissue across multiple morphological, biomechanical, biochemical, and cellular viability parameters. The results consistently show that the optimized protocol removes cellular components and nuclear material more effectively while better preserving ECM architecture, biomechanical integrity, and bioactive factors essential for nerve regeneration. Morphological assessments using H&E and DAPI confirm that the optimized method achieves near-complete decellularization without compromising ECM ultrastructure. This is evidenced by the absence of residual nuclear debris, intact basal lamina, aligned collagen fibrils, and preserved endoneurial conduits, which are critical for supporting axonal guidance. Comparison between the optimized protocol and Hudson's method reveals that, morphologically, Hudson's method retained fragmented DNA and disrupted endoneurial structures, whereas the optimized protocol achieved near-complete decellularization with intact ECM architecture. These histological findings are strongly supported by biochemical analyses showing significantly reduced DNA and cell protein content, along with greater retention of collagen and laminin than Hudson's method. The optimized protocol achieved greater removal of immunogenic components (96.9% vs 90.5% DNA reduction) while better preserving ECM components (collagen: 381.6 vs 355 µg/mg; laminin: 21 vs 17.4 ng/mg) and neurotrophic factors (NGF: 56.2 vs 22.4 pg/mg). The congruence of these data sets validates the efficacy of the optimized protocol in removing immunogenic cellular materials while maintaining scaffold integrity. Biomechanically, the optimized scaffolds exhibit tensile properties intermediate between those of native and Hudson-treated nerves, balancing strength and elasticity. The optimized scaffold showed greater tensile strength (2.3 MPa vs 1.62 MPa) and more physiologically relevant elasticity (Young's modulus 13.76 MPa vs 9.44 MPa) than Hudson's method, while avoiding the hyperelasticity seen in Hudson-treated nerves (66.6% vs 47.2% strain). This biomechanical profile aligns with the preserved ECM microstructure observed via histology, suggesting the scaffold maintains sufficient mechanical cues for *in vivo* nerve function and regeneration. The optimized protocol achieved greater removal of immunogenic components (96.9% vs 90.5% DNA reduction) while better preserving ECM components (collagen: 381.6 vs 355 µg/mg; laminin: 21 vs 17.4 ng/mg) and neurotrophic factors (NGF: 56.2 vs 22.4 pg/mg). Furthermore, the controlled degradation kinetics of the optimized scaffolds, slower than Hudson's but faster than native tissue, indicate a favorable balance between scaffold persistence and remodeling potential. The biochemical preservation of neurotrophic factors such as

NGF, BDNF, and GDNF further underscores the optimized protocol's advantage. Although these factors remain below native levels, their significant enrichment relative to Hudson's method suggests improved scaffold functionality that supports cellular survival and axonal growth. Cell viability assays corroborate this, showing that BM-MSCs cultured on optimized scaffolds had significantly higher viability (OD 0.58 vs 0.43 at 72 hr) and lower caspase-3 activity (0.172 vs 0.224 OD) than on Hudson scaffolds. These improvements across all parameters position the optimized protocol as a superior platform for peripheral nerve tissue engineering. Overall, the strong alignment and mutual reinforcement among morphological, biochemical, biomechanical, and cellular viability results provide compelling evidence that the optimized decellularization protocol offers a markedly improved scaffold platform. This integrated dataset highlights the critical importance of preserving ECM architecture and bioactive molecules while effectively eliminating immunogenic components to maximize regenerative potential in peripheral nerve repair.

The optimized protocol's superior performance comes from its balanced approach: mild enzymatic digestion (papain, 18 min) combined with gentle detergent treatment (CHAPS and Zwittergent 10), which removes cellular components while minimizing ECM damage. By contrast, Hudson's method uses stronger ionic detergents (sodium deoxycholate) for longer periods; although effective for decellularization, it causes greater disruption of ECM ultrastructure and loss of bioactive molecules (27-29). Preserving basal lamina integrity and collagen alignment is particularly critical, as these structures provide essential physical and biochemical cues for Schwann cell migration and axonal regrowth (30, 31). Disruption of these ECM elements, as observed in Sondell (32) and Hudson's method, likely compromises scaffold functionality by altering mechanical stiffness and topographical guidance, leading to suboptimal regenerative outcomes. In the present study, the short duration (18 min) and optimized concentration of papain (2 mg/ml) were selected to minimize proteolytic damage to ECM while effectively removing cellular components, as supported by the preserved histological architecture and ECM quantification. The near-complete removal of nuclear material and cell proteins in the optimized scaffolds reduces the risk of immune activation and chronic inflammation post-implantation (33, 34). Residual DNA fragments and cellular remnants are well-known triggers of host immune responses that can impede regeneration and cause graft rejection (34, 35). By eliminating these immunogenic components more thoroughly, the optimized protocol creates a more immunologically inert environment conducive to constructive tissue remodeling. Biomechanically, the optimized scaffold's intermediate Young's modulus and ultimate stress values suggest it retains sufficient tensile strength to withstand physiological loads while maintaining compliance that mimics native nerve elasticity (36). Excessive stiffness or hyperelasticity, as seen with Hudson's method, could disrupt mechanotransduction pathways critical for cellular behavior and axonal pathfinding. The optimized scaffold-controlled deformation capacity and reduced peak extension likely support the dynamic mechanical environment needed for nerve regeneration without imposing abnormal strain on regenerating axons or supporting cells (37). The degradation profile further complements scaffold

functionality by providing a temporally regulated matrix turnover (38, 39). The slower degradation rate compared to Hudson's method may prevent premature scaffold collapse, preserving structural support during early regeneration phases. At the same time, it degrades faster than native tissue, allowing gradual replacement by host ECM and cells, which is essential for successful integration and functional recovery (40). Retention of neurotrophic factors such as NGF, BDNF, and GDNF in the optimized protocol is a particularly notable advantage, as these molecules actively promote neuronal survival, differentiation, and axonal elongation. The partial preservation of these factors likely results from milder processing conditions that avoid excessive protein denaturation or washout (41). Although concentrations remain below native levels, the significant improvements over Hudson's method suggest enhanced biological signaling within the scaffold microenvironment, which likely contributes to improved cellular viability and reduced apoptosis in BM-MSC cultures (5, 42). However, the result underscores the need for continued methodological refinement to improve neurotrophin retention. The cellular viability and caspase-3 activity assays provide functional validation of the scaffold's biocompatibility. Enhanced metabolic activity and lower apoptotic signaling on optimized scaffolds indicate a more supportive niche for transplanted or endogenous stem/progenitor cells, which are crucial for orchestrating regenerative processes (43). This improved cellular response may stem from the combined effects of preserved ECM architecture, retained neurotrophic factors, and reduced immunogenic load, highlighting the multifactorial benefits of optimized decellularization. Mechanistic insights from this comprehensive dataset emphasize that effective peripheral nerve scaffold design requires a delicate balance: aggressive enough to remove immunogenic cellular components but gentle enough to preserve ECM structure, mechanical properties, and bioactive cues. The optimized protocol exemplifies this balance, yielding a scaffold that not only structurally resembles native nerve tissue but also supports cellular viability and regenerative signaling. These findings provide a strong foundation for future *in vivo* studies and clinical translation to improve peripheral nerve repair outcomes.

In summary, our optimized decellularization approach not only corroborates but also extends the current literature by more comprehensively preserving ECM structure, mechanical integrity, and bioactive components. These improvements collectively enhance scaffold biocompatibility and regenerative potential, positioning this protocol as a promising candidate for translational nerve repair applications.

Limitations

Although this study demonstrates the effectiveness of the optimized decellularization protocol, it has several notable limitations. First, the findings are primarily based on *in vitro* assessments, and the performance of the optimized scaffolds *in vivo* has not been verified; future research should evaluate these scaffolds in animal models to assess biocompatibility and regenerative efficacy in a physiological environment. Additionally, the authors did not thoroughly investigate the scaffolds' long-term stability and biodegradation kinetics, which is crucial for understanding their performance over extended periods. The cellular viability assays used BM-

MSCs, raising questions about how other cell types, such as Schwann cells or neurons, respond to the scaffolds. Furthermore, the study was limited to specific scaffold sizes and geometries, which may limit the generalizability of the results and necessitate further exploration of how these parameters influence scaffold performance. While biomechanical testing provided valuable insights, additional assessments, such as fatigue testing and dynamic loading scenarios, are essential to understand scaffold performance under physiological conditions fully. The degradation study was conducted in PBS, which mainly accounts for hydrolytic degradation. Future studies should evaluate enzymatic pathways (e.g., collagenase) to simulate physiological conditions. Lastly, the potential effects of UV-C sterilization on ECM protein integrity and mechanical properties were not evaluated separately. As the scaffolds were tested after sterilization, this represents the final product; however, future studies comparing pre- and post-sterilization properties would be valuable.

Future perspectives

The findings from this research open several promising avenues for future exploration and development. Conducting comprehensive *in vivo* studies will be critical to validating the effectiveness of the optimized scaffolds in promoting nerve regeneration, including assessments of functional recovery and long-term outcomes. Future work could also focus on enhancing scaffold properties by incorporating additional bioactive molecules or growth factors, potentially using surface modifications or drug-delivery systems for controlled release. Investigating personalized approaches, such as creating patient-specific scaffolds tailored to individual needs, could significantly improve clinical outcomes. Moreover, exploring combination therapies that integrate optimized scaffolds with strategies such as electrical stimulation or gene therapy may enhance functional recovery. Additionally, while this study focused on peripheral nerve repair, the optimized decellularization protocol could have broader applications in other areas of tissue engineering and warrants further investigation. Lastly, addressing regulatory pathways and conducting early-phase clinical trials will be essential to translating these findings into clinical practice and facilitating the transition from bench research to bedside application.

Conclusion

In summary, this study successfully demonstrates that the optimized decellularization protocol significantly enhances the preservation of ECM components, mechanical properties, and bioactive factors in nerve scaffolds. The results indicate that our approach not only maintains ECM structural integrity but also improves cellular viability and reduces apoptosis when seeded with BM-MSCs. These advancements are critical for promoting nerve regeneration and functional recovery in peripheral nerve injuries. Furthermore, the findings highlight the importance of scaffold design in facilitating favorable cellular interactions and biomechanical performance. While the study provides a promising foundation for future research, particularly *in vivo* evaluations and potential clinical applications, it also underscores the need to further explore personalized approaches and combination therapies. Ultimately, the optimized decellularization protocol holds significant promise for advancing nerve repair strategies and improving

outcomes in regenerative medicine.

Acknowledgment

University of Mohaghegh Ardabili, Iran, financially supports the present research. We are thankful for their support.

Ethics Approval

All experimental procedures involving rat and human peripheral nerve tissues were conducted in accordance with the EU Council Directive of 24 November 1986 and approved by the Ethics Committee of Mohaghegh Ardabili University, Iran. All protocols complied with institutional, national, and international guidelines for the ethical use of biological tissues.

Funding

The University of Mohaghegh Ardabili, Iran, financially supported this research.

Authors' Contributions

S Z and A Ab designed the experiments; H A and A As performed the experiments and collected data; S Z, A Ab, and A As discussed the results and strategy; H A prepared the draft manuscript and visualization. S Z, A Ab, and A As revised and edited the article. S Z and A Ab supervised, directed, and managed the study; S Z, A Ab, A As, and H A approved the final version for publication.

Conflicts of Interest

The authors declare no competing interests.

Declaration

The authors gratefully acknowledge the use of artificial intelligence (AI)-assisted language tools solely for improving grammar, clarity, and readability. Following the use of these tools, the manuscript was thoroughly reviewed and revised by all authors to ensure the accuracy of the scientific content, the validity of interpretations, and compliance with academic standards. AI tools were not used for data generation, data analysis, study design, conceptual development, or the production of the scientific content of the manuscript. The authors take full responsibility for the originality, integrity, and accuracy of the final version of the submitted manuscript.

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